Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

The Certification of the Mass Fractions of Stilbenes in Bovine Urine

Certified Reference Materials ERM®-BB386 and ERM®-BB389

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CERTIFICATION REPORT

The Certification of the Mass Fractions of Stilbenes in Bovine Urine

Certified Reference Materials ERM®-BB386 and ERM®-BB389

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Summary

This report describes the preparation of the pork meat matrix reference materials ERM-BB386 and ERM-BB389 and the certification of the content (mass fraction) of three stilbenes.

The preparation and processing of the material, homogeneity and stability studies, and the characterisation are described hereafter and the results are discussed. Uncertainties were calculated in compliance with the ISO/IEC Guide 98-3 Guide to the Expression of Uncertainty in Measurement (GUM) [1] and include uncertainties due to possible heterogeneity, instability, and characterisation. The certified values and their uncertainties are listed below:

ERM-BB389

Stilbenes in the reconstituted material	Certified value [2] [µg/kg]	Uncertainty [µg/kg]	Number of accepted sets of results
Diethylstilbestrol (DES) [1]	1.1	0.5[3]	5
Dienestrol (DE)	5.5	1.4[4]	6
Hexestrol (HEX)	6.1	0.9[4]	6

1) Sum of cis- and trans-isomer.
2) Mass fractions based on the unweighted mean of accepted results.
3) The uncertainties are the expanded uncertainties (k = 2.78) of the values defined in 2).
4) The uncertainties are the expanded uncertainties (k = 2.57) of the values defined in 2).

ERM-BB386

Stilbenes in the reconstituted material	Certified value [1] [µg/kg]
Diethylstilbestrol (DES)	< 0.6
Dienestrol (DE)	< 0.6
Hexestrol (HEX)	< 0.4

1) Given as CCβ of the most sensitive method of the characterisation study. With 95 % confidence, the true value of the material is below this value.

The assigned values and their uncertainties are based on a minimum sample intake of 1 g reconstituted material.

Table of contents

1 Glossary

ANOVA ..... Analysis of variance
b ..... Slope of the linear regression
BCR ..... Bureau Communautaire de Référence
C_{18} ..... Octadecyl silica stationary phase
CCα ..... Decision limit
CRM ..... Certified reference material
DE ..... Dienstilbestrol
DES ..... Diethylstilbestrol
ERM ..... European Reference Material
EURL ..... European Reference Laboratory
GC-MS ..... Gas chromatography-mass spectrometry
GUM ..... Guide to the Expression of Uncertainty in Measurement
HEX ..... Hexestrol
HFBA ..... Heptafluorobutyric anhydride
k ..... Coverage factor
IRMM ..... Institute for Reference Materials and Measurements
ISO ..... International Organization for Standardization
LC-MS ..... Liquid chromatography mass spectrometry
LC-MS/MS ..... Liquid chromatography-tandem mass spectrometry
LLE ..... Liquid liquid extraction
LOQ ..... Limit of quantification
MRM ..... Multiple reaction monitoring
MS ..... Mass spectrometry
MSD ..... Mass spectrometric detector
$MS_{between}$ ..... Mean of squares between groups (ANOVA)
MS_{within} ..... Mean of squares within groups (ANOVA)
MSTFA ..... N-Methyl-N-(trimethyl-silyl)trifluoroacetamide
n ..... Number of replicates
NRL ..... National Reference Laboratory
RSD ..... Relative standard deviation
RSD_{stab} ..... Relative standard deviation of all results of the stability study
s ..... Standard deviation
s_b ..... Standard deviation of the slope of the linear regression
s_{bb} ..... Between-bottle standard deviation
SI ..... International System of Units
SPE ..... Solid phase extraction
s_{wb} ..... Within-bottle standard deviation
$t_{\alpha,df}$ ..... Student t-value for a given probability and degrees of freedom
u^*_{bb} ..... Relative standard uncertainty due to the heterogeneity that can be hidden by the method repeatability
u_{bb} ..... Relative standard uncertainty due to between-bottle heterogeneity
u_{char} ..... Relative uncertainty of the characterisation exercise
u_{CRM} ..... Combined uncertainty of certified value
$u_{CRM, rel}$ ..... Combined relative uncertainty of certified value
U_{CRM} ..... Expanded uncertainty of certified value
$U_{CRM, rel}$ ..... Expanded, relative uncertainty of certified value
u_{lts} ..... Relative uncertainty of long-term stability
u_{sts} ..... Relative uncertainty of short-term stability
u_{meas} ..... Uncertainty of measurement result
UPLC ..... Ultra-performance liquid chromatography
u_{sts} ..... Relative uncertainty of short-term stability

u_{Δ} ..... Combined uncertainty of certified value and measurement uncertainty
U_{Δ} ..... Expanded uncertainty of certified value and measurement uncertainty
$v_{MSwithin}$ ..... Degrees of freedom of MS_{within}
x ..... Pre-defined shelf life
x_i ..... Time point i in an isochronous stability study
Δ ..... Difference between two measurement results
Δ_m ..... Difference between measured and certified value
$\bar{x}$ ..... Average of all time points in an isochronous stability study
$\bar{y}$ ..... Average of all results of a homogeneity study

2 Introduction

2.1 Background

Veterinary drugs are used to treat animal diseases or as prophylactic agents. However, they can also be abused, either applying them in a wrong way (e.g. too short withdrawal period) or in a non-authorised manner, as growth promoters. Many drugs exhibit besides their desired activity also adverse side effects, such as carcinogenicity. This constitutes a potential hazard for the consumer.
Several hundreds of pharmacologically active substances were and have been administered to food-producing animals for various purposes, the most important ones being cure and prevention from diseases. In contrast to these, one important class are compounds with hormonal activity to increase the animal growth rate. Stilbenes are important examples for the substances and show an anabolic effect similar to steroids.
DES (diethylstilbestrol) was the first synthetic estrogen to be created [2]. Never patented, DES was marketed by numerous drug companies using hundreds of brand names in the belief it prevented miscarriages and premature deliveries. It was later found to be carcinogenic and teratogenic and was phased out in the late 1970s. During the 1960s DES was used as a growth hormone in the beef and poultry industry. In the light of toxicological problems in humans, the use of stilbenes was banned as growth promoting agents in veal and calf production [3]. Stilbenes have been demonstrated to be highly potent carcinogenic compounds [4].
European legislation stipulates sampling and monitoring plans [5], and establishes Community and National Reference Laboratories (EURLs/NRLs) and their tasks and responsibilities. Three main types of (certified) RMs for veterinary drug testing can be distinguished: incurred matrix materials (incorporation of the drug in the animal during a controlled feeding study), blank matrix materials (matrices with proven “absence” of a given analyte), and calibrants (pure substances with proven identity and purity). Current monitoring programmes include stilbenes in their lists and despite their classification as group A1 (banned) substances, their use in animal feeding has to be monitored.
The candidate materials are preparations of new materials to replace the current stilbene-containing CRMs BCR-389 (diethylstilbestrol, DES), BCR-390 (dienestrol, DE), and BCR-391 (hexestrol, HEX) and the respective blank materials BCR-386, BCR-387, BCR-388. As all three compounds can be chromatographically separated, IRMM decided to produce a single "stilbene positive" material containing DES, DE and HEX and a single "stilbene negative" blank material. Target concentrations were lowered to 1 µg/kg - 6 µg/kg per substance. The volume per unit is raised to 5 mL per unit (previously 2 mL) to allow for more replicates per unit to be taken.

2.2 Choice of the material

An incurred material which closely resembles a typical sample analysed in the laboratory in terms of comparable analyte extractability was considered necessary. The blank and incurred materials were obtained in collaboration with the Rijksinstituut voor Volksgezondheid en Milieu (NL) and the Centraal Veterinair Instituut van Wageningen (NL). Urines were collected from a 5 month old calf. The blank and the incurred urine samples were collected before and after administration of the individual stilbenes, respectively. After the collection of 25 L of blank urine, DES, DE and HEX were administered separately as a concentrated solution (8 mg/mL in plant oil) by intramuscular injection in three cycles. Each study cycle consisted in the collection of urine after the injection of the respective stilbene. The urine samples were checked for their concentrations and were sent frozen to IRMM.

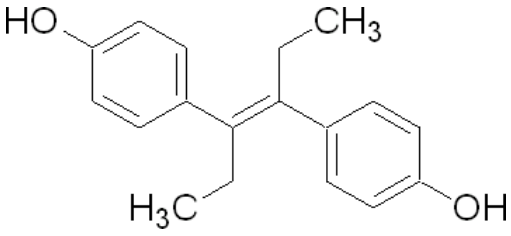
Figure 1 depicts the parent drugs whose absence in ERM-BB386 and presence in ERM-BB389 was confirmed.

2.3 Definition of analytes and chemical structures

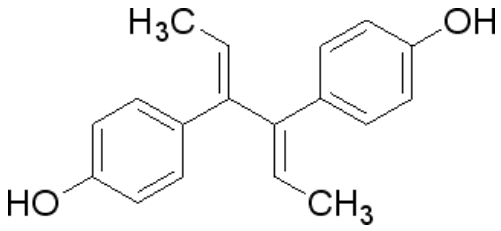
Table 1. Definition of stilbene analytes comprised in ERM-BB389.

Trivial name and abbreviation	IUPAC name	CAS number	Chemical formula	Molecular mass (g/mol)
Diethylstilbestrol (DES)	4,4'-(3E)-hex-3-ene-3,4-diyldiphenol	56-53-1	$C_{18}H_{20}O_2$	268.35
Dienestrol (DE)	4-[4-(4-hydroxyphenyl)hexa-2,4-dien-3-yl]phenol	84-17-3	$C_{18}H_{18}O_2$	266.33
Hexestrol (HEX)	4-[4-(4-hydroxyphenyl)hexan-3-yl]phenol	84-16-2	$C_{18}H_{22}O_2$	270.37

DES



DE



HEX

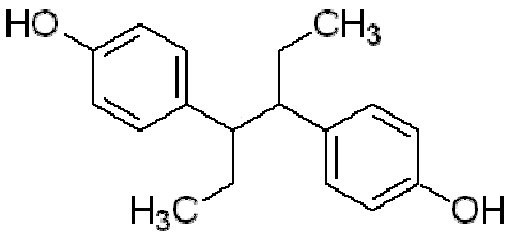


Fig. 1: Chemical structures of the three stilbenes present in ERM-BB389.

3 Participants

Project management and evaluation:
European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Reference Materials Unit, BE
(Work performed under ISO Guide 34 accreditation; BELAC 268-TEST)

Raw material provision:
Rijksinstituut voor Volksgezondheid en Milieu, NL

Centraal Veterinair Instituut van Wageningen, NL
(Work performed under ISO 9001 certification; 12097-2006-AQ-ROT-RvA)

Processing:
European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Reference Materials Unit, BE
(Processing performed under ISO Guide 34 accreditation; BELAC 268-TEST)

Homogeneity and stability measurements:
C.E.R. Groupe, Laboratoire d'Hormonologie, BE
(Measurements performed under ISO/IEC 17025 accreditation; BELAC 073-TEST)

Characterisation analysis:
Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, DE
(Measurements performed under ISO/IEC 17025 accreditation; AKS-PL-12005)

C.E.R. Groupe, Laboratoire d'Hormonologie, BE
(Measurements performed under ISO/IEC 17025 accreditation; BELAC 073-TEST)

CLVCE – Central Laboratory of Veterinary Control and Ecology, BG
(Measurements performed under ISO/IEC 17025 accreditation; BAS 61 LI)

GD – Gezondheidsdienst voor Dieren Deventer, NL
(Measurements performed under ISO/IEC 17025 accreditation; RvA L120)

Laboratori Agroalimentari, Generalitat de Catalunya, ES
(Measurements performed under ISO/IEC 17025 accreditation; ENAC 157/LE309)

NMVRVI – National Food and Veterinary Risk Assessment Institute, LT
(Measurements performed under ISO/IEC 17025 accreditation; DAP-PL-3328.99)

RIKILT – Instituut voor Voedselveiligheid, NL
(Measurements performed under ISO/IEC 17025 accreditation; RvA L014)

ÚSKVBL – Institute for State Control of Veterinary Biologicals and Medicaments, CZ
(Measurements performed under ISO/IEC 17025 accreditation; CAI 621/2007)

4 Processing of the material

Raw materials were provided by RIVM (Rijksinstituut voor Volksgezondheid en Milieu, NL); preparation of the incurred and the blank urine was done at the Centraal Veterinair Instituut (Wageningen, NL). The urines were derived from a 5 month old calf; the incurred urines were collected after intramuscular injections of the individual substances.
The urines used for processing consisted of approximately 15 L of blank bovine urine and 0.5 L of each of the incurred urines DES, DE and HEX. These urines were checked for the concentration of stilbenes prior to processing. The three incurred urines were mixed and diluted with the blank urine.

For ERM-BB389, 3 x 10 mL of the incurred urines were centrifuged for 5 min at 10000 rpm. Aliquots of the spiked urines were placed in a 100 mL volumetric flask and made up to volume with blank urine. The incurred stilbene solution was diluted in 6.9 L blank urine to yield 7 L of spiked urine. For ERM-BB386, the blank urine was directly used after centrifugation.

The dilution of the stilbenes resulted in a single material containing all three substances (ERM-BB389). The concentration of DES around 1 µg/kg is close to the detection/quantification limits of many laboratories (see also chapter 7.2). This level can help laboratories to improve their measurement capabilities in this very low, but important concentration range. The concentrations of DE and HEX were chosen at levels from 5 µg/kg to 6 µg/kg, reflecting typical concentrations in the middle range of typical calibration ranges.

The mixed urine was placed on a magnetic stirring plate and aliquots of 5 mL were dispensed into 30 mL vials using an ampouling machine. Argon was used to flush the head space of the vial before and after filling. The variation in the dispensed volume was determined gravimetrically with twenty vials and the masses of the dispensed urine were determined as 5.179 ± 0.004 g (ERM-BB389) and 5.200 ± 0.004 g (ERM-BB386), respectively.

The filled vials were placed in a pre-cooled freeze dryer flushed with nitrogen. Each material (ERM-BB386 and ERM-BB389) was dried separately to avoid cross-contamination of the blank material. A freeze drying programme developed during a previous feasibility study was used. Due to the fact that urine is a complex matrix with high salt content and organic molecules present, the initial shelf temperature was -50 °C to avoid melting of the material during the initial steps of the drying process. The shelf temperature was raised to -30 °C. Thereafter the freeze-drying programme with duration of about 5 days was started with the typical sequence: freezing, sublimation, and secondary drying. After the secondary drying step was finished, the chamber was flushed with nitrogen to provide an inert head space. After freeze-drying, the vials were capped and labelled.

The water content in the final material was measured by Karl Fischer titration [6]. Five bottles of the batch were chosen using a random stratified sample picking scheme and analysed in duplicate. The results are summarized in Table 2.

Table 2. Water content

Material	mean ± s [g/kg]
ERM-BB386	45.1 ± 2.8
ERM-BB389	44.6 ± 2.9

5 Homogeneity study

5.1 Homogeneity

For the homogeneity study, 10 samples of ERM-BB389 were chosen using a random stratified sample picking scheme and analysed in quadruplicate for their stilbene content. Measurements were performed by a validated GC-MS method in the multiple reaction monitoring mode (MRM). Calibration was done with neat standard solutions, and deuterated internal standards were spiked to the samples in the beginning of the extraction procedure.

Samples were measured in a random order (predefined at IRMM and communicated to the laboratory) to allow distinction between an analytical trend and a trend in the filling sequence. Measurements were performed under repeatability conditions. In all ERM-BB389 samples, the three stilbenes were identified and quantified.

Data were checked for single and double outliers by applying the Grubbs test at a confidence level of 95% and 99%. One outlier was detected (DE, analytical sequence, Double Grubbs test, 99% level) which was scrutinised and retained as no technical reason was found to eliminate it. Regression analysis was performed to detect possible trends regarding the filling sequence or analytical sequence.

No significant slopes were found at 95 % or 99 % level. In conclusion, the material can be regarded as homogeneous for all three stilbenes. Furthermore it was checked whether the data followed a normal or unimodal distribution using normal probability plots and histograms, respectively. Individual data and sample averages showed a unimodal distribution for all analytes, although some deviations from normality were observed. Finally, the uncertainty contribution from possible heterogeneity was estimated by a one-way analysis of variance (ANOVA) [7]:

Method repeatability (s_{wb}) expressed as a relative standard deviation is given as follows:

$$s_{wb} = \frac{\sqrt{MS_{within}}}{\bar{y}}$$

MS_{within}: mean square within a bottle from an ANOVA
$\bar{y}$: average of all results of the homogeneity study

Between-unit variability (s_{bb}) expressed as a relative standard deviation is given by the following equation:

$$s_{bb} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}}$$

$MS_{between}$: mean square among bottles from an ANOVA
n: average number of replicates per bottle

The heterogeneity that can be hidden by method repeatability is defined as follows:

$$u_{bb}^{*} = \frac{s_{wb}}{\sqrt{n}} \sqrt[4]{\frac{2}{v_{MSwithin}}}$$

$v_{MSwithin}$: degrees of freedom of MS_{within}

The larger value of s_{bb} or u^{*}_{bb} was used as uncertainty contribution for homogeneity, u_{bb} (see Table 3 for a summary of results, values were converted into relative uncertainties).

Table 3. Homogeneity study results for ERM-BB389

	DES	DE	HEX
RSD [%]	1.217	1.570	1.211
s_{wb} [%]	2.670	3.110	3.351
s_{bb} [%]	n.c.	0.217	n.c.
u^*_{bb} [%]	0.678	0.790	0.851
u_{bb} [%]	**0.678**	**0.790**	**0.851**

n.c. = not calculable because $MS_{between} < MS_{within}$

5.2 Minimum sample intake

Within-bottle heterogeneity is closely correlated to the minimum sample intake. Due to the intrinsic heterogeneity, individual subsamples of a material may not contain the same amount of analyte. The smallest subsample that still is representative for the complete bottle is the minimum sample intake. The larger the intrinsic heterogeneity, the larger is the minimum sample intake.
1 g was the sample intake used in the homogeneity and stability studies. The minimum sample intake therefore is 1 g of reconstituted material, proving that the samples are homogeneous at least at this level. This was also confirmed by results from the characterisation study.

6 Stability studies

6.1 Short-term stability study

A four weeks isochronous study [8] was performed to evaluate stability of ERM-BB389 during transport. Thirty samples were selected from the produced batch using a random stratified sampling scheme. Samples were dispatched to the testing laboratory on dry ice.

Samples were stored at +4 °C, +18 °C, +60 °C and at a reference temperature of -70 °C. Three ampoules were stored at each temperature for 0, 1, 2, and 4 weeks. After the indicated storage periods, the samples were transferred to storage at -70 °C until analysis. Samples were analysed in triplicate under intermediate precision conditions (the analytical sequence lasted two days) in the order predefined at IRMM (randomised sample order) using the same GC-MS method as for the homogeneity study.

Data (Annex B) were first checked for single and double outliers by applying the Grubbs test at confidence levels of 95% and 99%, respectively. No outliers were detected. Data points were plotted against time and the regression lines were calculated (see Table 4 for a summary). The observed slopes were tested for significance using a t-test, with $t_{\alpha,df}$ being the critical t-value (two-tailed) for a confidence level of 95 %. The slope was considered as statistically significant when $b/s_b > t_{\alpha,df}$. In one case (DE), slopes significantly different from 0 were found for storage temperatures at +18 ºC and +60 °C. It was shown that the uncertainty of the short-term stability (u_{sts}) is negligible if sample shipment is carried out under cooled conditions, which therefore shall be the dispatch condition for sample shipment to the customer.

Table 4. Evaluation of the short-term stability study

	DES			DE			HEX		
Statistical parameters	4 ºC	18 ºC	60 ºC	4 ºC	18 ºC	60 ºC	4 ºC	18 ºC	60ºC
$\|b\|/s_b$	0.85	0.65	0.16	0.82	2.98	3.62	0.70	1.19	1.13
Statistical significance (95% conf. interval) [1]	No	No	No	No	Yes	Yes	No	No	No
u_{sts} [%/week]	0.37	0.30	0.34	0.31	0.32	0.61	0.54	0.43	0.43

[1] $|t_{0.05;\,34}| = 2.03$

6.2 Long-term stability study

A 24 months isochronous study [8] was performed to evaluate the stability of ERM-BB389 during storage. The chosen study duration was a suitable compromise between data for sound statistics and considering stability data from the previous batch indicating suitable analyte stability over various years at -20 ºC.

Twenty samples were picked from the produced batch using a random stratified sampling scheme. Samples were stored at -20 °C, and at a reference temperature of -70 °C. 4 vials were stored at each temperature for 0, 6, 12, 18, and 24 months, respectively. After storage at -20°C for the indicated periods, the samples were transferred to -70 °C until analysis. Samples were dispatched on dry ice and kept at -20 ºC in the laboratory until analysis. Samples were analysed in triplicate under repeatability conditions in the order predefined at IRMM (randomised sample order) using the same GC-MS method as for the homogeneity study.

Data (Annex C) were checked for single and double outliers by applying the Grubbs test at confidence levels of 95% and 99%, respectively. No outliers were detected.

Data points were plotted against time and the regression lines were calculated to check for significant trends (degradation, enrichment) due to storage conditions. The observed slopes were tested for significance using a t-test, with $t_{\alpha,df}$ being the critical t-value (two-tailed) for a confidence level of 95 %. The slope was considered as statistically significant when $b/s_b > t_{\alpha,df}$.

Finally, the uncertainty of stability u_{lts} [9] was calculated for a pre-defined period of 2 years as:

$$u_{lts} = \frac{RSD_{stab}}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot x$$

with RSD_{stab} being the relative standard deviation of all individual results of the relevant stability study, x_i being the time point for each replicate, $\bar{x}$ being the average of all time points and x being the pre-defined shelf life. Results are summarized in Table 5.

Table 5. Evaluation of the long-term stability study

	DES	DE	HEX
Statistical parameters	-20 ºC	-20 ºC	-20 ºC
$\|b\|/s_b$	1.00	1.667	0.667
Statistical significance (95% conf. interval)[1]	No	No	No
u_{lts} [%/2 years]	1.308	3.351	2.765

[1] $|t_{0.05;\,58}| = 2.00$

7 Characterisation

7.1 Design of the study

GC-MS and LC-MS methods were applied for the characterisation of the reference material as the vast majority of testing laboratories are applying these techniques for confirmatory stilbene analysis.

Eight laboratories were carefully selected to perform the analytical measurements. Validated methods and a quality system were an indispensable requirement for participation; a method under an accreditation scope was considered an asset. The laboratories had to prove their measurement capabilities and had to demonstrate previous experience in stilbene analysis in comparable matrices.

The sample preparation principle was similar in all laboratories, although the method parameters varied considerably from laboratory to laboratory (see Table 7).

The laboratories used their own calibrants of different sources which were verified for their purity. All laboratories used internal standards to quantify the analytes.

The certified value for DES was determined as the total of the trans- and the cis-isomer. Laboratories were asked to provide optional data for both isomers, if possible. Out of the accepted data sets, only one laboratory provided concentrations for the individual isomers.

For the characterisation of ERM-BB386 and ERM-BB389, each laboratory was provided with the following samples:

- 2 units of ERM-BB386
- 2 units of ERM-BB389
- 1 vial of BCR-391 (hexestrol in bovine urine) as a control sample

Laboratories had to apply their validated methods; calibration solutions were prepared by the participant laboratories according to the laboratories' method working instructions (neat standard solution calibration or matrix-matched calibration). Measurements had to be performed on two different days with independent calibrations on each day. Each of the two samples had to be measured four times, whereby quadruplicate measurements for each sample had to be done on both days. Reconstitution of the samples was prescribed as follows: to one vial of the candidate material, 4.84 g (ERM-BB386) or 4.87 g (ERM-BB389) of distilled water had to be added and homogeneised according to the instructions given by IRMM. Some laboratories have a high sample intake and needed one entire vial for one replicate measurement; they were provided with eight instead two vials.

7.2 Results and technical evaluation

The individual methods employed by the laboratories are summarised in Tables 6 - 8 (sample preparation and calibration, separation, transitions used for quantification). It can be seen that the laboratory methods varied substantially in terms of employed extraction solution and clean-up procedure. The determinations also differed in the separation principle LC and GC), the type of derivatisation in GC-MS and the type and source of the calibrants used. The standards used for calibration were obtained from five different sources. Chromatographic conditions, columns and equipment were also different. All laboratories applied mass spectrometric detection, but using different mass transitions and detectors.

Table 6. Methods in the characterisation study – sample preparation and calibration

Lab code	Sample intake [g]	Deconjugation/ Extraction	Clean-up	Derivatisation	Calibration	Internal standard
1	5	25 µL beta-glucuronidase *E. coli* 1 h, 55°C, pH 7	Detectabuse column (Biochemical diagnostics)	-	7 points, 0-10 µg/kg	6-alpha-fluoro prednisolone
2	1	50 µL beta-glucuronidase overnight, 37°C, pH 5.4	SPE (OASIS + Amino)	-	5 points, 0-25 µg/kg	DES-d6
3	1	20 µg beta-glucuronidase Helix Pomatia overnight, 37°C, pH 5.0; LLE with diethylether	SPE C_{18} and immunoaffinity chromatography	MSTFA	6 points 0-3 µg/kg DES 0-8 µg/kg DE, HEX	DES-d6, HEX-d4
4	2	20 µL beta-glucoronidase/aryl-sulphatase 16 h, 37°C, pH 4.8	SPE (C_{18} + Amino)	MSTFA	6 points, 0-10 µg/kg	DES-d6, DE-d2, HEX-d4
5	1	beta-glucuronidase Helix Pomatia overnight, 37°C, pH 5.2 LLE with TBME	SPE (C_{18} + Amino)	HFBA	7 points, 0-3 µg/kg	DES-d6
6	5	beta-glucuronidase Helix Pomatia 2 h, 55°C, pH 5.0 LLE with hexane	SPE (C_{18} + Amino)	MSTFA	4 points, 2.5-15 µg/kg	DES-d6
7	5	beta-glucuronidase Helix Pomatia 16 h, 37°C, pH 5.2; LLE with diethylether	SPE (OASIS + Amino)	-	9 points, 0-10 µg/kg	DES-d6
8	5	beta-glucuronidase/aryl-sulphatase overnight, room temperature, pH 5.2	Immunoaffinity column (Randox)	HFBA	6 points, 0-20 µg/kg	DES-d8, DE-d2, HEX-d4

Some laboratories used deuterated DES as only internal standard; others used deuterated standards for all analytes. Five different internal standards were applied in total by the laboratories (see last column of Table 6). The individual results as obtained are listed in Annex D.

Table 7. Methods in the characterisation study – separation and quantification

Lab code	LC or GC column	Solvent system/GC injector	Chromatographic system	Mass spectrometer
1	Atlantis C_{18}, 100 x 2.1 mm, 3 µm (Waters)	Acetonitrile/acetic acid/ ammonium acetate	Waters Acquity UPLC	Quattro Premier (Micromass)
2	Acquity UPLC BEH C_{18}, 100 x 2.1 mm, 1.7 µm (Waters)	Acetonitrile/Ammonium acetate	Waters Acquity UPLC	Quattro Ultima (Micromass)
3	Agilent DB5MS, 30 m, 0.25mm (0.25 µm)	300°C, splitless	Agilent	Quattro Micro (Micromass)
4	Restek Rtx-CLPesticides 30 m, 0.25mm (0.25 µm)	250 °C, splitless	Agilent 6890N	Quattro Micro (Waters)
5	Restek Rxi-5Sil, 30 m, 0.25mm (0.25 µm)	260°C, splitless	Agilent 6890N	Agilent 5975 inert XL MSD
6	VF-5 ms Varian, 30 m, 25 mm (25 µm)	260°C, splitless	Thermo Finnigan	Trace MS (Finnigan)
7	Luna® $C_{18\ (2)}$, 150 x 2mm, 5 µm (Phenomenex)	Acetonitrile/methanol/ water	Agilent 1100	4000 Q-trap (Applied Biosystems)
8	Gemini® C_{18}, 150 x 3 mm, 5 µm (Phenomenex)	80°C, cool on-column	Agilent 6890	Agilent 5973 Inert MSD

Table 8. Methods in the characterisation study – mass traces and transitions used for quantification

Lab code	DES	DE	HEX
1	267>251	265>236	269>134
2	267>251	265>93	269>134
3	412>383	410>395	207>179
4	412>217	410>395	207>179
5	660[1]	658[1]	331[1]
6	412[1]	410[1]	207[1]
7	267>237	265>93	269>119
8	660[1]	658[1]	331[1]

[1] Single quad MS

After receipt of the data sets, the results were subjected to technical evaluation. The average of all data sets for the control sample (12.6 µg/kg) was very close to the certified value (13.3 µg/kg) of this material (BCR-391). However, two results were very different from the rest: although the average of laboratory 4 differed seemingly, the results agreed within the respective uncertainties [10]. As this was not the case for lab 1, all data sets for laboratory 1 were not considered for evaluation. Laboratory 6 had not analysed the samples according to the pre-established measurement protocol given by IRMM (sample reconstitution, number of replicates). Consequently, all data sets for laboratory 6 were not considered for evaluation. Laboratory 2 reported no values for DES (below LOQ). In laboratory 5, two values on the first measurement day were excluded for technical reasons. The laboratory itself had expressed its doubts about these values as some technical problems were observed during the analysis of these samples.

In total, 38 results for DES from 5 laboratories, and 46 results each for DE and HEX from 6 laboratories were accepted after technical scrutiny and subjected to statistical data assessment.

The results of the statistical tests of the finally considered data for ERM-BB389 are summarized in Table 9. It shall be noted that the mean of means (certified value) hold for the reconstituted material. Normality plots do not indicate a deviation from the normal distribution.

Table 9. Summary of statistical evaluation for ERM-BB389

Analyte	DES	DE	HEX
Number of data sets	5	6	6
Number of replicate measurements	38	46	46
Mean of means [µg/kg]	1.11	5.46	6.11
Relative standard deviation of mean of means [%]	30.73	22.04	11.42
Relative standard error of mean of means [%]	13.74	9.00	4.66
Outlying means? (Dixon test, Nalimov t-test, Grubbs test)	No	Lab 4	Lab 4

The outlying means of DE and HEX (lab 4) are not significantly different from the mean of means if the measurement uncertainties instead standard deviations of the characterization measurements are taken into consideration [10].

The results for ERM-BB386 were reported as "not detected" by all laboratories that provided acceptable data sets. Only data from laboratories that had provided acceptable data sets for the evaluation of ERM-BB389 were considered. Initially, laboratories 5 and 6 reported the detection of stilbene peaks in this blank material. During technical scrutiny, it was found that the reported value was below the LOD of laboratory 5 and that contradicting peaks, retention times and rather noisy chromatograms obtained in single quad mode in the second lab can be attributed to artifacts and/or contamination in laboratory 6. Finally, results from five laboratories were accepted.

Table 10. LOQ and CCβ values for DES, DE and HEX in the methods of the characterization study (after technical scrutiny).

Lab	LOQ DES [µg/kg]	CCβ DES [µg/kg]	LOQ DE [µg/kg]	CCβ DE [µg/kg]	LOQ HEX [µg/kg]	CCβ HEX [µg/kg]
2	1.0		1.0		1.0	
3	0.5		0.5		0.5	
7		0.82		0.90		0.92
8		0.6		0.6		0.4

Table 10 lists the limits of quantification (LOQ) or CCβ values of the laboratory methods for the three stilbenes (values have been determined according to 2002/657/EC [11]). The calculated CCβ of 0.1 µg/kg of lab 4 is likely an underestimation as the value was determined by using the noise of a blank. The laboratory agreed on this point and admitted that higher CCβ values would be more appropriate. As the determination of this certified value should only be based on reliable data, the values of laboratory 4 were not taken into account.
The CCβ is the concentration at and above which it can be concluded with an error probability of 5% that the candidate material would be tested as a 'compliant', i.e. a stilbene negative sample. It was decided to take the lowest CCβ values from laboratory 8 (DES and DE: 0.6 µg/kg; HEX: 0.4 µg/kg) as the certified value.

8 Certified values and uncertainties

The certified values for ERM-BB389 are calculated as the mean of means of the accepted data sets. The standard error of the mean of means was used as an estimation of the uncertainty contribution of the characterisation exercise. The standard error is calculated as the standard deviation divided by the square root of the number of accepted data sets.
The combined uncertainty of the certified value includes contributions from the between- bottle heterogeneity, long-term storage, and the characterisation study. The relative combined uncertainty is calculated as the square root of the sum of squares of the relative uncertainties of the individual contributions, according to:

$$u_{CRM} = \sqrt{u_{bb}^2 + u_{lts}^2 + u_{char}^2}$$

Table 11 summarizes the individual uncertainty contributions and the resulting expanded uncertainties, and indicates the certified values and their uncertainties after rounding. Coverage factors were chosen according to the t-distribution depending of the number of accepted sets of results. This approach was chosen due to the low number of available data sets.

Table 11. Certified values and uncertainties for ERM-BB389

	DES[1)]	DE	HEX
u_{bb} [%]	0.678	0.790	0.851
u_{lts} [%][2)]	1.308	3.351	2.765
u_{char} [%]	13.74	8.998	4.662
$u_{CRM, rel}$ [%]	13.82	9.634	5.487
$U_{CRM,rel}$ (k = 2.78, *f* = 4) [%]	38.38		
$U_{CRM,rel}$ (k = 2.57, *f* = 5) [%]		24.77	14.11
Certified value [µg/kg]	**1.1**	**5.5**	**6.1**
U_{CRM} [µg/kg]	**0.5**	**1.4**	**0.9**

1) Sum of cis- and trans-isomer.
2) Shelf life 24 months.

The certified values for ERM-BB386 correspond to the CCβ of the most sensitive method in the characterisation study that provided an acceptable data set (Table 12):

Table 12. Certified values for ERM-BB386

	DES[1)]	DE	HEX
Certified value [µg/kg]	**< 0.6**	**< 0.6**	**< 0.4**

1) Sum of cis- and trans-isomer.

9 Metrological traceability

DES, DE and HEX are structurally defined molecules. The measurements results for assigning stilbene mass fraction values to the material were obtained by employing methods with different sample preparation procedures (from deconjugation under different conditions to extraction with organic solvent with subsequent clean-up using different conditions and stationary phases). Laboratories used gas chromatography and liquid chromatography for analyte separation in combination with mass spectrometry for quantification. The chromatography parts of the methods mainly differed in type of mobile phases used, the type of separation columns applied (stationary phases, particle sizes, column dimension), and chromatography system differences (flow rate, column temperature, injected sample amount, elution conditions). Similar MS traces or MS/MS transitions (parent ions, daughter ions) were used for quantification but MS methods differed in some compound-dependent parameters (dwell times, collision energies). Calibrants from different sources were used for the calibrations.

For the obtained data, independence of the results from the extraction technique can be concluded. As all laboratories used mass spectrometric detection, certified values only hold when mass spectrometry is employed for quantification. Only validated methods were used. Correctness of calibration was confirmed by agreement of results with a CRM used as a quality control sample.

Consequently, the certified mass fractions for DES, DE and HEX are traceable to the International System of Units (SI).

10 Instructions for use

10.1 Safety precautions

The usual laboratory safety precautions apply.

10.2 Reconstitution of the material

- Allow the bottle to warm up to ambient temperature before opening.
- ERM-BB386: Add accurately 4.84 ± 0.01 g of distilled water to the content of the vial. The weighing should be performed immediately after opening of the vial to minimise water uptake by the lyophilised powder.
- ERM-BB389: Add accurately 4.87 ± 0.01 g of distilled water to the content of the vial. The weighing should be performed immediately after opening of the vial to minimise water uptake by the lyophilised powder.
- Close the vial and mix to a homogeneous sample, for instance by vortexing the powder-water mixture for at least 1 min at maximum speed.

The analysis of the material should be started in less than 1 hour after reconstitution.

10.3 Intended use

This material is intended to be used for method performance control and validation purposes (trueness determination). For the assessment of the method performance, the measured values of the CRMs are compared with the certified values following a procedure [10] described here in brief:

- Calculate the absolute difference between mean measured value and the certified value (Δ_m).
- Combine measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_\Delta = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_Δ) from the combined uncertainty (u_Δ) using a coverage factor of two ($k = 2$), corresponding to a confidence interval of approximately 95 %

- If $\Delta_m \leq U_\Delta$ then there is no significant difference between the measurement result and the certified value, at a confidence level of about 95 %.

The material is not intended to be used with immunochemical methods.

10.4 Storage conditions

The materials should be stored at a temperature of -20 ± 5°C. However, the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises.

11 Acknowledgements

The authors would like to thank R. Zeleny and T. Linsinger (IRMM, BE) for internal review of this report, and E. Nordkvist (National Veterinary Institute, SE), P. Finglas (Institute of Food Research, UK), S.P.J. van Leeuwen (Institute for Environmental studies, VU Amsterdam, NL) as Certification Advisory Panel members for reviewing the certification documents and for their constructive comments.

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Annex A. Homogeneity data

Table A1. Results of homogeneity study

Bottle number	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Replicate 3 (µg/kg)	Replicate 4 (µg/kg)	Average (µg/kg)
	DES				
72	0.59	0.57	0.60	0.58	0.59
129	0.59	0.63	0.58	0.58	0.60
305	0.61	0.56	0.61	0.61	0.60
441	0.60	0.59	0.60	0.59	0.60
533	0.58	0.59	0.59	0.58	0.59
639	0.59	0.58	0.58	0.58	0.58
756	0.58	0.57	0.60	0.58	0.58
809	0.57	0.61	0.56	0.58	0.58
954	0.58	0.62	0.61	0.59	0.60
1073	0.59	0.59	0.60	0.58	0.59
	DE				
72	5.30	5.28	5.41	5.34	5.33
129	5.20	5.74	5.19	5.24	5.34
305	5.29	5.15	5.50	5.39	5.33
441	5.32	5.17	5.36	4.95	5.20
533	5.30	5.39	5.49	5.53	5.43
639	5.18	5.47	5.12	5.37	5.29
756	5.79	5.24	5.34	5.47	5.46
809	5.27	5.39	5.16	5.14	5.24
954	5.15	5.32	5.30	5.39	5.29
1073	5.29	5.28	5.35	5.00	5.23
	HEX				
72	6.90	6.64	6.25	6.61	6.60
129	6.79	6.32	6.56	6.68	6.59
305	6.50	6.38	6.77	6.06	6.43
441	6.33	6.61	6.57	6.37	6.47
533	6.63	6.61	6.69	6.77	6.68
639	6.74	6.76	6.46	6.52	6.62
756	6.27	6.75	6.34	6.44	6.45
809	6.73	6.19	6.78	6.49	6.55
954	6.61	6.32	6.58	6.58	6.52
1073	6.85	6.57	6.10	6.60	6.53

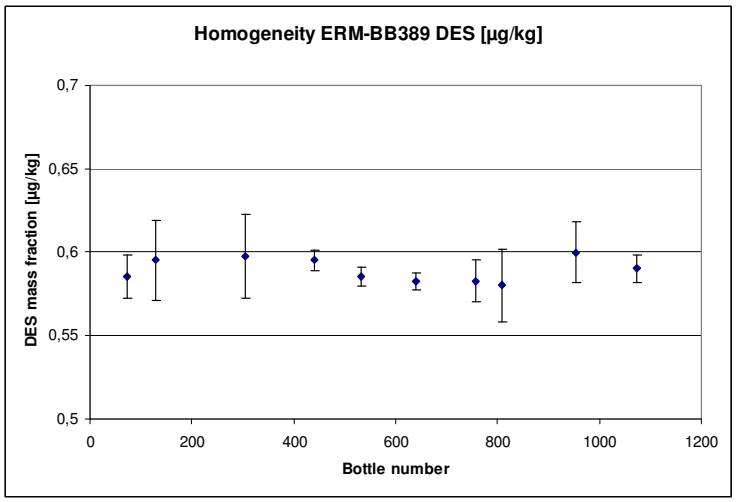

Figure A1. Homogeneity of DES in ERM-BB389. The x axis depicts the sample numbers (filling sequence). The indicated points are mean values of quadruplicate measurements.

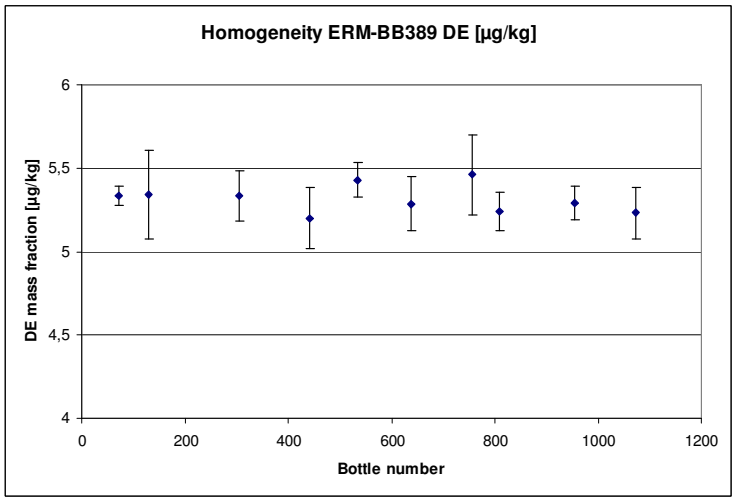

Figure A2. Homogeneity of DE in ERM-BB389. The x axis depicts the sample numbers (filling sequence). The indicated points are mean values of quadruplicate measurements.

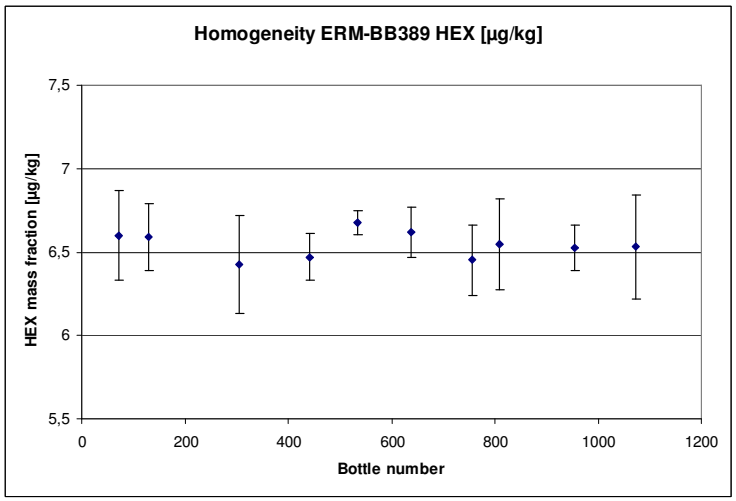

Figure A3. Homogeneity of HEX in ERM-BB389. The x axis depicts the sample numbers (filling sequence). The indicated points are mean values of quadruplicate measurements.

Annex B. Short-term stability data

Table B1. Results of the short-term stability study

Time (weeks)	4 ºC	18 ºC	60 ºC	4 ºC	18 ºC	60 ºC	4 ºC	18 ºC	60 ºC
	DES			**DE**			**HEX**		
0	0.61	0.61	0.61	6.03	6.03	6.03	6.58	6.58	6.58
0	0.58	0.58	0.58	5.63	5.63	5.63	6.54	6.54	6.54
0	0.57	0.57	0.57	5.75	5.75	5.75	6.58	6.58	6.58
0	0.56	0.56	0.56	5.76	5.76	5.76	6.15	6.15	6.15
0	0.58	0.58	0.58	5.91	5.91	5.91	6.64	6.64	6.64
0	0.57	0.57	0.57	5.48	5.48	5.48	6.29	6.29	6.29
0	0.61	0.61	0.61	5.81	5.81	5.81	6.65	6.65	6.65
0	0.57	0.57	0.57	6.02	6.02	6.02	5.68	5.68	5.68
0	0.60	0.60	0.60	6.22	6.22	6.22	6.56	6.56	6.56
1	0.56	0.57	0.59	5.86	5.82	5.06	6.37	6.44	6.03
1	0.6	0.59	0.58	5.72	5.75	5.27	6.42	6.13	6.43
1	0.57	0.57	0.57	5.66	5.73	5.49	6.09	6.48	6.33
1	0.59	0.59	0.58	5.76	5.89	5.91	6.50	6.10	6.45
1	0.59	0.56	0.60	6.09	5.84	5.50	6.65	5.73	6.27
1	0.59	0.57	0.58	5.66	5.73	5.48	6.36	6.35	6.49
1	0.63	0.61	0.58	6.19	6.06	5.64	6.51	6.36	5.93
1	0.58	0.58	0.56	5.92	5.59	5.16	6.34	6.31	6.13
1	0.57	0.58	0.62	5.81	5.74	5.36	6.34	6.50	6.75
2	0.62	0.60	0.59	5.87	5.60	5.37	6.54	6.48	5.99
2	0.57	0.58	0.58	5.82	5.68	5.38	6.47	6.09	6.34
2	0.57	0.60	0.57	5.75	5.53	6.06	6.20	6.32	6.29
2	0.59	0.58	0.62	6.05	5.78	5.58	6.30	6.23	6.22
2	0.58	0.57	0.57	5.82	5.79	5.04	6.08	5.84	6.14
2	0.59	0.57	0.61	5.83	5.72	5.36	6.44	6.06	6.37
2	0.58	0.59	0.59	5.98	6.06	5.30	6.08	6.33	6.06
2	0.60	0.58	0.58	6.00	5.59	5.12	5.10	6.35	6.26
2	0.59	0.58	0.56	5.78	5.74	4.99	6.10	6.22	6.57
2	0.60	0.58	0.60	5.70	5.54	5.69	6.40	6.33	6.13
2	0.58	0.58	0.61	5.93	5.71	5.57	6.44	6.28	6.12
4	0.58	0.58	0.59	5.84	5.57	5.55	6.37	6.18	6.18
4	0.57	0.60	0.58	5.78	5.75	5.18	6.41	6.45	6.34
4	0.60	0.57	0.58	5.61	5.49	5.04	6.51	5.94	6.40
4	0.63	0.57	0.58	5.73	5.80	5.53	5.94	6.23	6.55
4	0.62	0.60	0.58	5.91	5.83	4.99	6.15	6.43	6.21
4	0.58	0.58	0.57	5.76	5.53	5.08	6.41	6.03	6.53
4	0.56	0.54	0.55	5.82	5.44	5.02	6.44	6.44	6.02
4	0.61	0.61	0.61	6.03	6.03	6.03	6.58	6.58	6.58
4	0.58	0.58	0.58	5.63	5.63	5.63	6.54	6.54	6.54

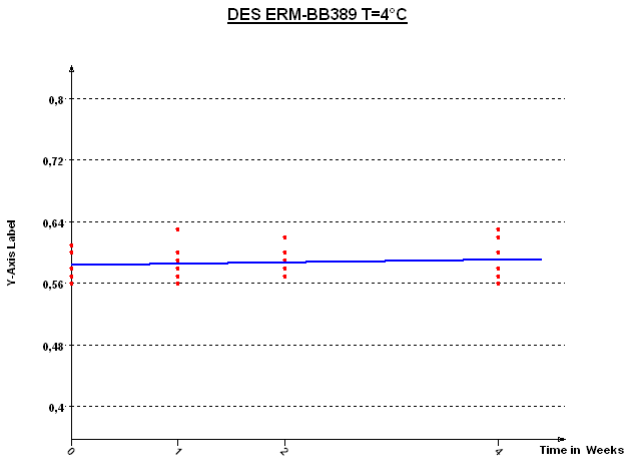

Figure B1. Short-term stability for DES at 4 ºC.

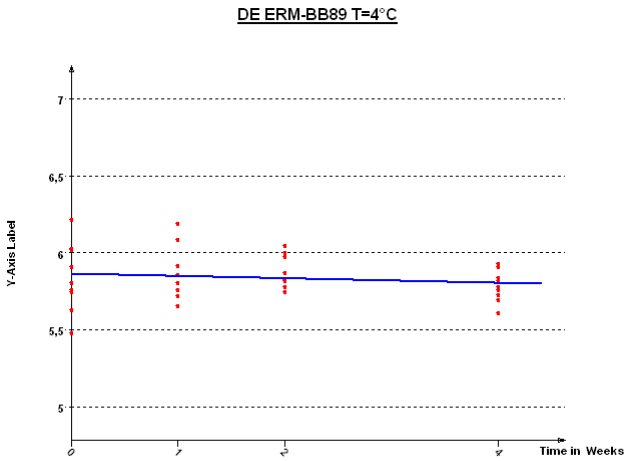

Figure B2. Short-term stability study for DE at 4 ºC.

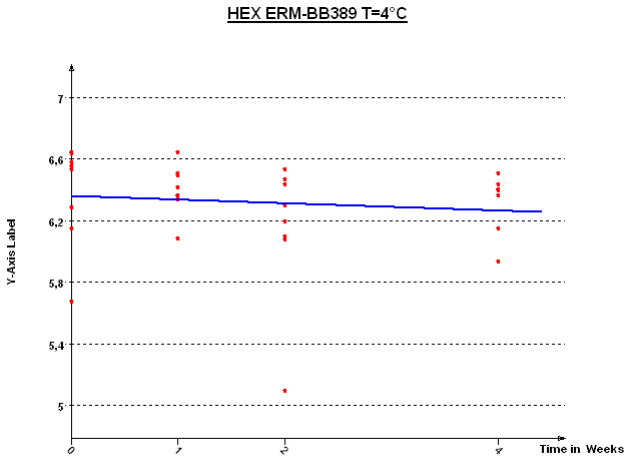

Figure B3. Short-term stability for HEX at 4 ºC.

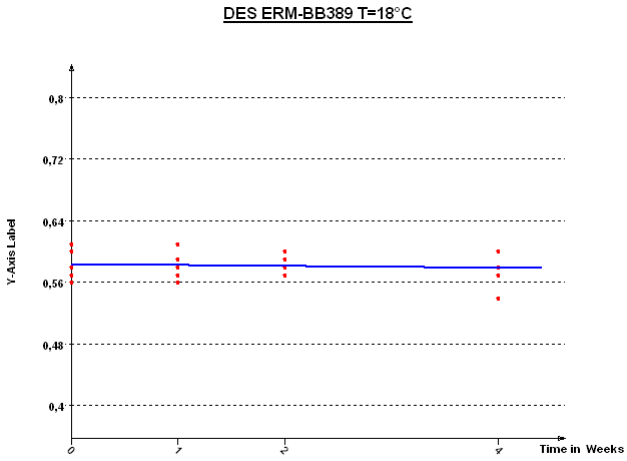

Figure B4. Short-term stability for DES at 18 ºC.

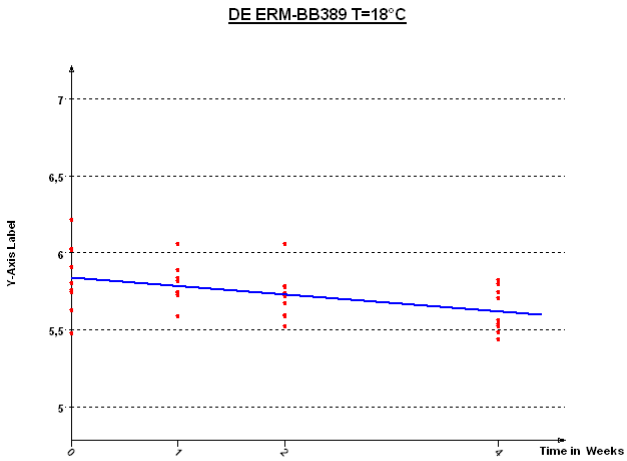

Figure B5. Short-term stability for DE at 18 ºC.

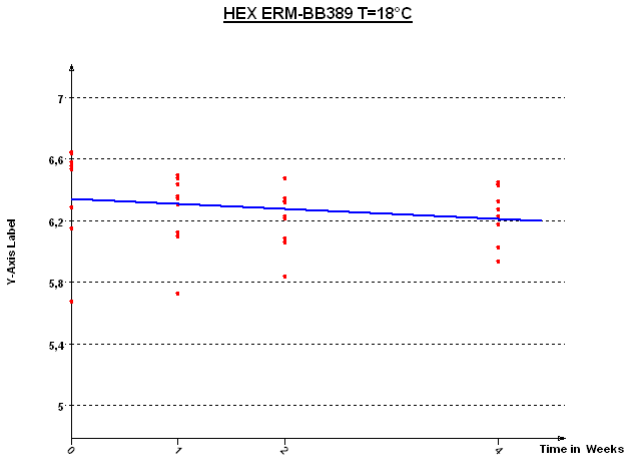

Figure 6. Short-term stability for HEX at 18 ºC.

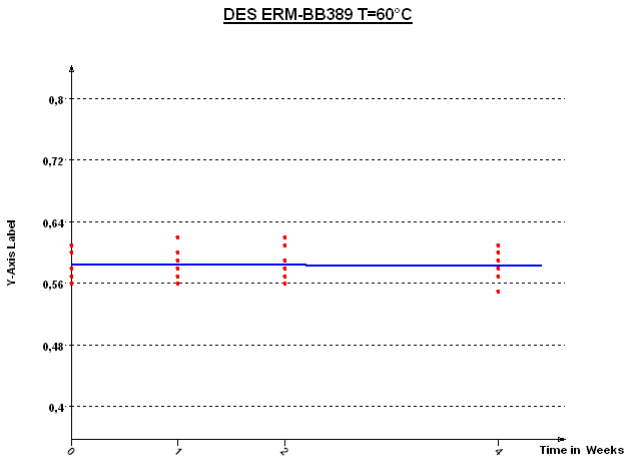

Figure B7. Short-term stability for DES at 60 ºC.

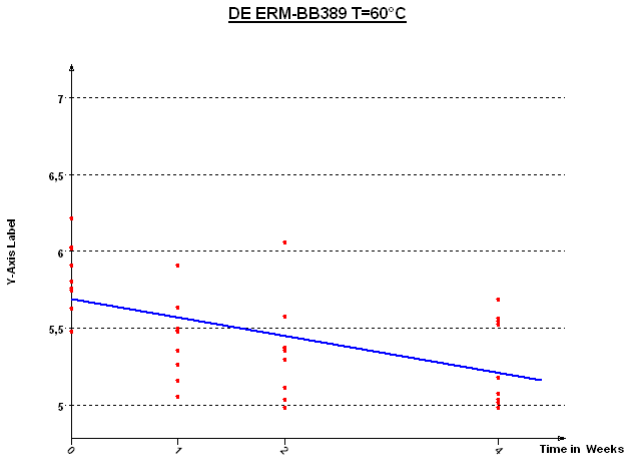

Figure B8. Short-term stability for DE at 60 ºC.

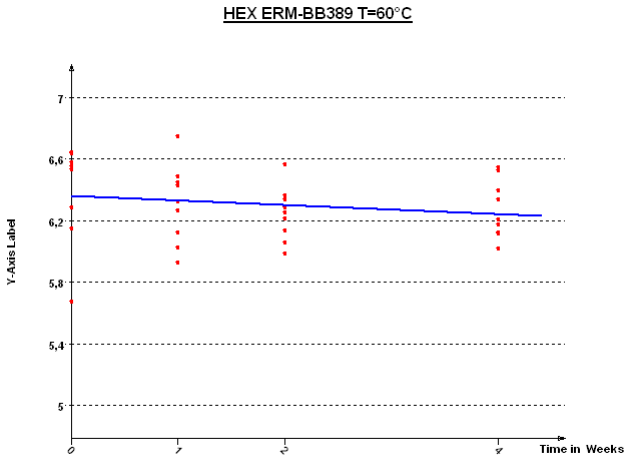

Figure B9. Short-term stability for HEX at 60 ºC.

Annex C. Long-term stability data

Table C1. Results of the isochronous long-term stability studies at -20 ºC.

Time (months)	DES	DE	HEX
0	1.02	7.34	5.82
0	1.12	7.45	5.68
0	1.08	7.46	4.81
0	1.11	7.10	5.68
0	1.09	7.15	5.67
0	1.12	7.13	5.20
0	1.02	7.08	5.08
0	1.01	7.27	5.32
0	1.01	6.97	5.13
0	1.06	7.27	5.95
0	1.13	7.67	5.35
0	1.11	7.56	5.91
6	1.08	7.10	5.40
6	1.06	7.25	5.70
6	1.04	7.36	5.03
6	1.10	7.09	5.58
6	1.07	7.00	5.20
6	1.13	7.12	4.42
6	1.07	7.67	5.99
6	1.06	2.53	5.45
6	1.05	7.51	5.98
6	1.09	7.11	4.60
6	1.06	7.12	5.84
6	1.10	7.25	5.65
12	1.11	7.50	5.37
12	1.11	7.45	6.00
12	1.08	7.33	5.94
12	1.12	7.11	4.60
12	1.11	7.12	5.84
12	1.02	7.25	5.65
12	0.99	6.80	4.75
12	1.05	7.19	5.25
12	1.05	7.07	5.90
12	1.04	7.22	5.73
12	1.08	7.20	6.09
12	1.08	7.28	5.99
18	1.11	7.16	6.08
18	1.08	7.21	5.91
18	1.08	7.05	6.15
18	1.09	7.31	5.55
18	1.06	7.08	4.77
18	1.05	7.15	5.80
18	1.12	6.97	5.26
18	1.06	7.16	5.84
18	1.16	7.11	5.34
18	0.98	6.40	5.49
18	1.06	6.79	5.36
18	1.06	6.64	5.20
24	1.03	7.43	6.06
24	1.06	7.16	5.97
24	0.97	7.02	5.73
24	1.07	7.15	5.40
24	1.07	6.91	5.59
24	1.07	6.96	5.26
24	1.03	6.40	5.49
24	1.05	6.79	5.36
24	1.08	6.64	5.20
24	1.03	7.22	5.66
24	1.14	7.22	5.94
24	1.07	7.35	6.09

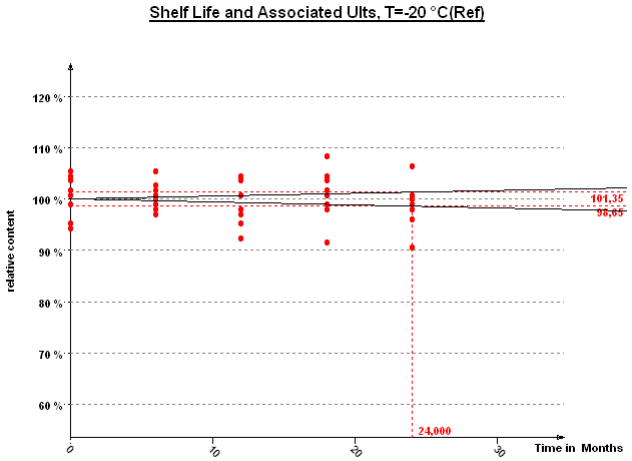

Figure C1. Long-term stability for DES at -20 ºC with associated u_{lts} for a storage period of 24 months.

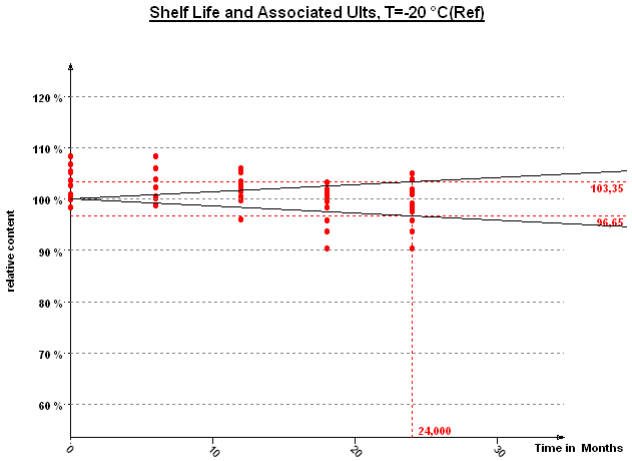

Figure C2. Long-term stability for DE at -20 ºC with associated u_{lts} for a storage period of 24 months.

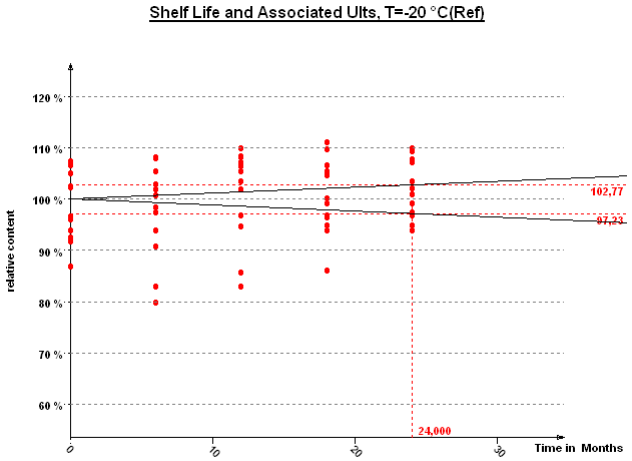

Figure C3. Long-term stability for HEX at -20 ºC with associated u_{lts} for a storage period of 24 months.

Annex D. Characterisation data

Table D1. Results of characterisation measurements for DES

DES mass fraction in ERM-BB389 [µg/kg]								
Lab code	Day 1				Day 2			
3-GC-MS	0.83	0.96	0.85	0.84	0.75	0.78	0.78	0.8
4-GC-MS	1.57	1.83	1.85	1.66	1.23	1.45	0.94	1.41
5-GC-MS	1.05	1.05	*	*	1.14	1.18	1.07	1.56
7-LC-MS	1.53	1.38	1.43	1.4	1.25	1.27	1.36	1.32
8-GC-MS	0.84	0.86	0.72	0.69	0.65	0.56	0.48	0.8

* excluded for technical reasons

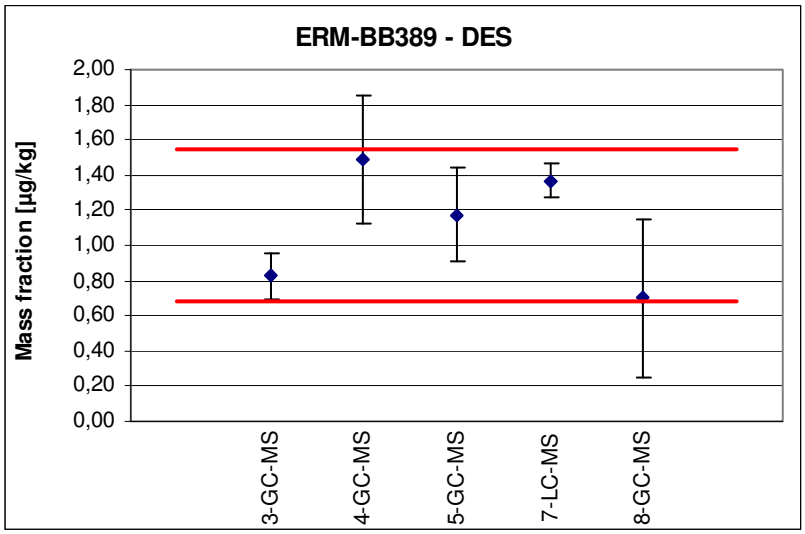

Figure D1. Laboratory means, mean of means and their uncertainties for DES. Red lines represent the expanded uncertainty of the CRM.

Table D2. Results of characterisation measurements for DE

DE mass fraction in ERM-BB389 [µg/kg]								
Lab code	Day 1				Day 2			
2-LC-MS	5.2	4.9	5.2	4.5	4.2	3.4	4.2	3.2
3-GC-MS	6.51	5.67	5.23	6.36	5.9	5.94	5.85	5.92
4-GC-MS	9.26	8.79	9.13	8.17	6.4	7.25	5.41	6.86
5-GC-MS	4.6	4.38	*	*	4.8	4.71	4.64	5.27
7-LC-MS	4.92	4.78	4.74	4.89	4.89	4.74	4.81	4.89
8-GC-MS	5.8	5.6	5.3	4.9	5.1	4.7	5.6	5.1

* excluded for technical reasons

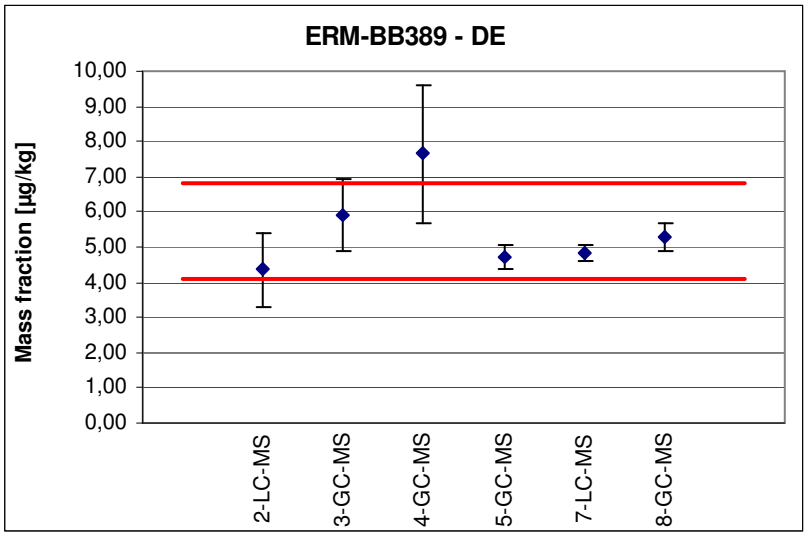

Figure D2. Laboratory means, mean of means and their uncertainties for DE. Red lines represent the expanded uncertainty of the CRM.

Table D3. Results of characterisation measurements for HEX

HEX mass fraction in ERM-BB389 [µg/kg]								
Lab code	Day 1				Day 2			
2-LC-MS	7.3	6.3	5.3	6.8	5.3	5.9	5.2	5.5
3-GC-MS	6.06	5.38	5.46	5.64	5.41	5.69	5.48	5.75
4-GC-MS	8.76	8.91	10.8	7.47	6.09	6.87	4.7	6.38
5-GC-MS	5.72	5.4	*	*	6.05	5.99	5.77	6.48
7-LC-MS	5.8	5.6	5.41	5.65	5.69	5.79	5.75	5.88
8-GC-MS	5.6	6.1	5.8	5.9	6.6	6.5	5.5	5.9

* excluded for technical reasons

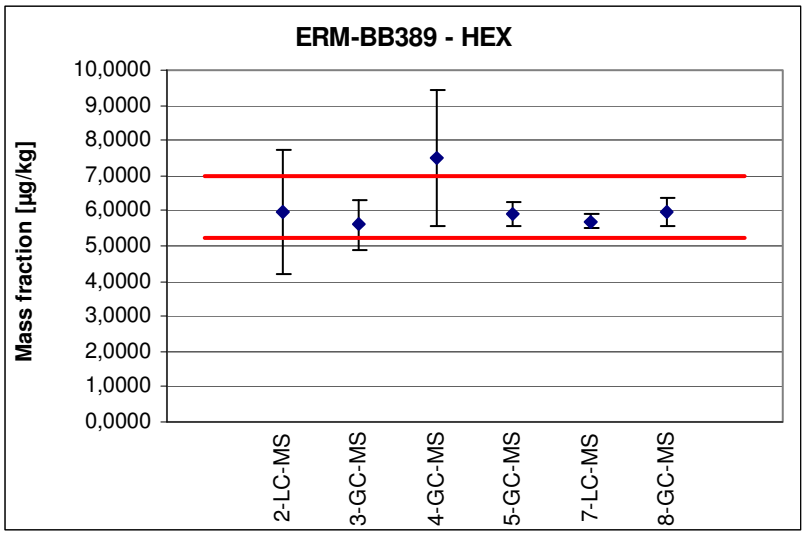

Figure D3. Laboratory means, mean of means and their uncertainties for HEX. Red lines represent the expanded uncertainty of the CRM.

European Commission

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Title: The Certification of the Mass Fractions of Stilbenes in Bovine Urine - Certified Reference Materials ERM®-BB386 and ERM®-BB389
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Abstract

This report describes the preparation of the pork meat matrix reference materials ERM-BB386 and ERM-BB389 and the certification of the content (mass fraction) of three stilbenes.

The preparation and processing of the material, homogeneity and stability studies, and the characterisation are described hereafter and the results are discussed. Uncertainties were calculated in compliance with the ISO/IEC Guide 98-3 Guide to the Expression of Uncertainty in Measurement (GUM) [1] and include uncertainties due to possible heterogeneity, instability, and characterisation. The certified values and their uncertainties are listed below:

ERM-BB389

Stilbenes in the reconstituted material	Certified value [2] [µg/kg]	Uncertainty [µg/kg]	Number of accepted sets of results
Diethylstilbestrol (DES) [1]	1.1	0.5[3]	5
Dienestrol (DE)	5.5	1.4[4]	6
Hexestrol (HEX)	6.1	0.9[4]	6

1) Sum of cis- and trans-isomer.
2) Mass fractions based on the unweighted mean of accepted results.
3) The uncertainties are the expanded uncertainties (k = 2.78) of the values defined in 2).
4) The uncertainties are the expanded uncertainties (k = 2.57) of the values defined in 2).

ERM-BB386

Stilbenes in the reconstituted material	Certified value [1] [µg/kg]
Diethylstilbestrol (DES)	< 0.6
Dienestrol (DE)	< 0.6
Hexestrol (HEX)	< 0.4

1) Given as CCβ of the most sensitive method of the characterisation study. With 95 % confidence, the true value of the material is below this value.

The assigned values and their uncertainties are based on a minimum sample intake of 1 g reconstituted material.

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